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ARTICLE

Axial control of protein reserve mobilization during germination of indian bean (*Dolichos lablab* L.) seeds

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ABSTRACT The influence of embryonic axis and exogenously applied plant growth hormones on protein mobilization and development of proteases have been investigated in Indian bean (*Dolichos lablab* L. var lignosus) seeds during germination and post-germinative growth up to 10 days. Accumulation of free amino acids synchronized with rapid proteolysis and higher levels were maintained throughout the germination period. The presence of proteases (acid, neutral and alkaline) with three different pH optima increased in the early stages of germination and decreased later. The axis-excision affected the activities of proteases and protein degradation. Furthermore, the free amino acid content increased continuously in detached cotyledons throughout the germination period. Treatment with 1% casein hydrolysate to simulate the accumulation of free amino acids had a telling inhibitory effect on the proteases in attached and detached cotyledons. Exogenously applied phytohormones BA (Benzyl adenine), GA_3 (Gibberellic acid) or IAA (Indole acetic acid) resulted in stimulation of development of proteases as well as proteolysis in detached cotyledons. The two hypotheses, source-sink and hormonal stimulus both were influencing in the mobilization of food reserves and the growth of seedling. The results of the study supports the role of axis in protein mobilization regulating the development of proteases by providing phytohormone signals and regulation of their activity *in vivo* by a feedback mechanism.

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KEY WORDS

Dolichos lablab
proteolytic enzymes
embryonic axis
plant hormones
protein reserve mobilization
seed germination

The mobilization of storage proteins is one of the most important post-germinative event in the growth and development of seedling. During germination period, the storage proteins are degraded by a variety of proteases which convert the insoluble storage proteins into soluble peptides and free amino acids; these are mobilized to the embryonic axis to support its growth and also provide energy by oxidation of the carbon skeleton after deamination (Mayer and Poljakolf-Mayber 1982; Bewley and Black 1994; Shutov and Vaintraub 1987; Okamato and Minamikawa 1998; Muntz et al. 2001; Schlereth et al. 2001). The food reserve mobilization and its regulation in dicotyledonous seeds has been receiving attention and two hypotheses have been put forth to explain the role of axis in the process. First, the growing axis may act as *sink* to draw away the products of degradation, which may inhibit further development of enzymes and/or inhibit their activities. Second, the growing axis may produce the plant growth substance(s) that stimulate the synthesis of hydrolytic enzymes needed for food reserve mobilization in the cotyledons (Davies and Slack 1981; Bewley and Black 1994; Nandi et al. 1995). The mobilization of food reserves and the growth of seedling appear to be an efficiently synchronized process with embryonic axis influencing the two processes.

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In cereals, the proteases responsible for the degradation of storage proteins are synthesized in aleurone layer and the synthesis of the enzymes is upregulated by gibberellins and down regulated by ABA (Abscisic acid; Rogers et al. 1985; Ritchie et al. 2000). However, the role of the embryo or embryonic axis in the control of food mobilization in dicotyledonous seeds is less understood. There are only a few reports regarding the protein reserve mobilization, which is influenced by either source-sink process or hormonal stimulus and no reports are available for the operation of two processes. The mechanisms of axial control need to be examined thoroughly to arrive at a unified mechanism of axial control of reserve mobilization in dicotyledons and require extensive studies with different species. Hence, the present study is aimed at investigating the mobilization of storage proteins and the role of embryonic axis in the regulation of protein mobilization during germination of Indian bean (*Dolichos lablab* L. var lignosus) seeds. We reported in this study the role of axis in protein mobilization regulating the development of proteases by providing phytohormone signals (hormonal stimulus) and the regulation of their activity *in vivo* by feed backmechanism (source-sink).

Materials and Methods

Indian bean (*Dolichos lablab* L. *var lignosus*) seeds were

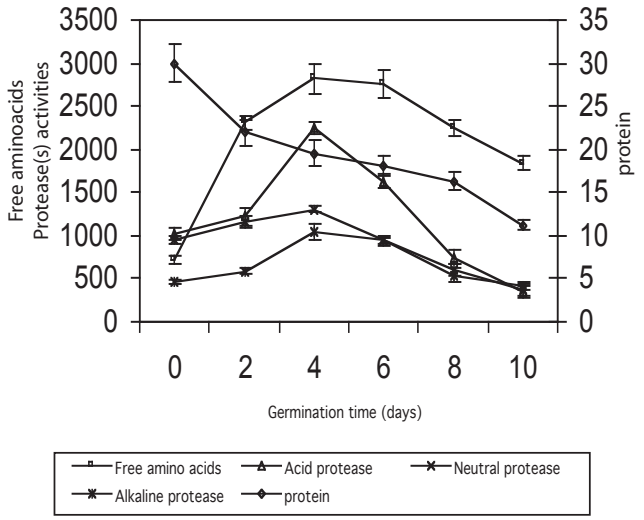

Figure 1. Changes in protein, free amino acid and the activities of acid, neutral and alkaline proteases in the cotyledons during the germination of Indian bean seeds.

procured from the Agricultural form of Andhra Pradesh Agricultural University, Rekulakunta, Anantapur, Andhra Pradesh, India. Healthy seeds of uniform size and weight were sorted and stored in a sterile plastic container until use. Seeds were surface sterilized with 0.1% $HgCl_2$ solution for 5 min and rinsed thoroughly with sterile distilled water. Soaking the seeds in distilled water for 12 h carried out imbibition. The water-imbibed seeds were germinated at room temperature for 10 days in sterile Petri dishes lined with moist filter paper. Sterile conditions were maintained by including 20 ppm of streptomycin sulphate in the incubation medium. Seedlings were withdrawn at designated time intervals and the cotyledons were carefully dissected out for analysis. The period of incubation (germination) was measured from the time when the imbibed seeds were transferred to the Petri dishes. Each experiment was carried out at least five times and each analysis was carried out in duplicate and averaged, unless otherwise stated.

To evaluate the influence of axis, the seed coat was removed after imbibitions and the cotyledons were separated from each other so that the axis remained attached to one of them. The cotyledons with (attached) or without (detached) axis were incubated with water or test solution under the same conditions.

Determination of free amino acids

An extract was prepared by boiling the cotyledons with 80% ethanol for 10 min. and centrifuged at 3,000 rpm for 10 min and the residue was re-extracted twice with hot alcohol and the pooled supernatants were filtered. The filtrate was suitably diluted and used for the estimation of amino acids by ninhydrin method (Raghuramulu et al. 2003).

Preparation of cotyledonary extract

The cotyledons were ground thoroughly in a pre-chilled mortar with chilled 0.05 M tris-HCl buffer, pH 7.2, containing 2 mM ß-mercaptoethanol. The extract was filtered and centrifuged at 10,000 rpm for 15 min. The supernatant was used for the estimation of proteins and assay of proteolytic enzymes. The results were expressed as µg/2 cotyledons.

Estimation of proteins

Protein content in the cotyledonary extract was estimated by the method of Lowry (1951). The results were expressed as mg/2 cotyledons.

Assay of proteolytic enzymes

Endopeptidase enzyme activity was measured by the modified method of Beevers (1968) using casein as substrate. The reaction mixture containing 1 ml of diluted enzyme extract, 1 ml of 1% casein (prepared in 0.1 N NaOH, pH adjusted to 7.0 with 0.1 N HCl), and 1 ml of appropriate buffer (0.1 M acetate buffer, pH 5.5, for acid protease, 0.1 M phosphate buffer, pH 7.3, for neutral protease and 0.025 M borate buffer, pH 8.8, for alkaline protease). Incubation was carried out for 1 h at 40°C. The reaction was arrested by the adding 1 ml of 20% trichloroacetic acid. The contents of the tube were kept at 4° C for 15 min and centrifuged at 3,000 rpm for 15 min. An aliquot of the supernatant was used for the determination of amino acids by ninhydrin method. The results were expressed as µmoles of amino acids released per hour in 2 cotyledons under experimental conditions.

Statistical analysis

Each value presented in figures and tables represent the arithmetic mean ± SE of five independent determinations, unless otherwise stated. The level of significance in between germination periods was calculated by DMR (Duncan Multiple Range) test.

Results

The maximal rates of protein depletion were observed during the first and last stages of germination (Fig. 1). Accumulation of free amino acids synchronized with rapid proteolysis and maximal rate of increase was observed in the first four days (250%, 30%) and then decreased marginally in between 2 to 15%. However, the level of free amino acids was still higher on day 10 by 2.5 fold, as compared to basal level on day 0. The cotyledonary extract of germinating Indian bean exhibited presence of caseinolytic activity at three different pH optima – 5.5, 7.3 and 8.8 (data not shown) – and the developmental profile of these three proteases (acid, neutral and alkaline) is depicted in Figure 1. The activities of all the three proteases increased uniformly up to day 4 and then

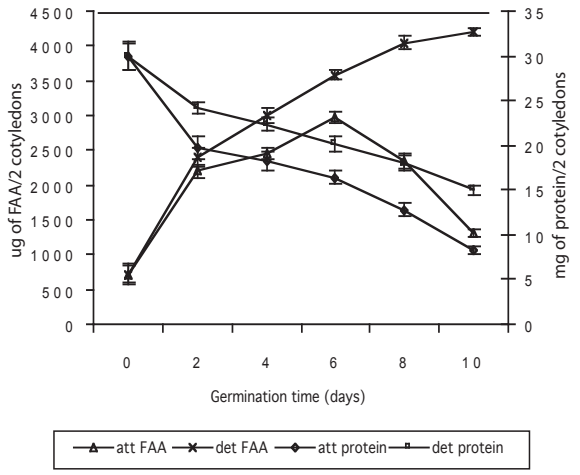

Figure 2A. Changes in protein and free amino acids in attached and detached cotyledons of Indian bean during germination.

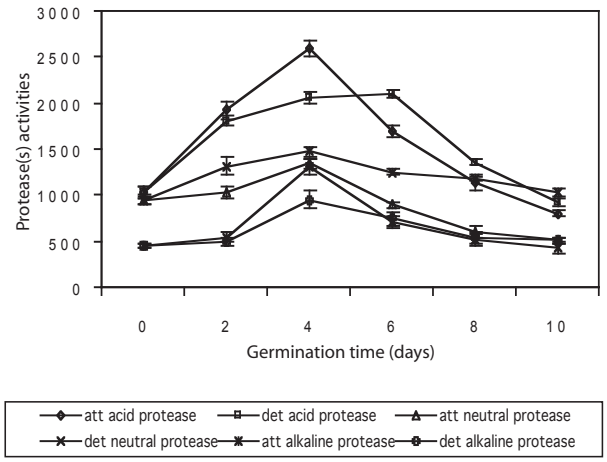

Figure 2B. Changes in proteases (acid, neutral and alkaline) in attached and detached cotyledons of Indian bean during germination.

gradually fell. However, the activity of acid proteases was higher throughout the germination period compared with the activities of neutral and alkaline proteases.

Role of axis on protein degradation

The influence of axis on protein degradation and proteases development was evaluated by using attached and detached cotyledons. Concomitant with the fall in the protein content, the free amino acid level in attached cotyledons increased maximally (3 fold) by day 2 and continued to increase up to day 6 and then declined further (Figure 2A). By contrast, a slower rate of degradation of protein was observed in detached cotyledons with maximal rates (16-20%) between day 0 and 2. The overall loss of protein in detached cotyledons was only 45% of the basal level while in attached cotyledons it was 72%. The free amino acid level in detached cotyledons, unlike in attached cotyledons, continued to increase throughout the germination period. It is pertinent to note that in the absence of embryonic axis the protein degradation retarded with accumulation of free amino acids.

The gradual development of all the three proteases in attached cotyledons increased up to day 4 and declined thereafter (Fig 2B). However, the developmental activities of all proteases in detached cotyledons were slow and delayed and maximal activity of acid protease observed on day 6 was nearly 20% lesser than the maximal activity observed on day 4 in attached cotyledons.

End product regulation of protein degradation

The possibility of the accumulation of free amino acids, end products of proteolysis, might bring about a repression of enzyme synthesis and/or inhibit the activities of proteases by feedback mechanism was examined. Germination in the presence of casein hydrolysate retarded the proteolytic process in attached and detached cotyledons compared to the cotyledons incubated in the absence of casein hydrolysate (Table 1). However, the accumulation of free amino acids was higher and the protein degradation was lower in detached cotyledons. The developments of all the three proteases were greatly affected and the activities on day 10 were one-third to one-fourth of the basal level in the presence of caseinhydrolysate.

Effect of exogenously applied plant growth hormones on protein degradation

Exogenously applied plant hormones (BA, GA_3 or IAA) had profound effect on protein degradation in detached cotyledons. Maximum stimulatory effect on the protease(s) activities in detached cotyledons was observed at concentrations of 0.01 mM of BA, GA_3 or IAA (Fig. 3), although perceptible changes were observed between 0.001 to 0.1 mM. All the hormones at these concentrations enhanced protein degradation as reflected by decrease in protein and increase in free amino acid level in detached cotyledons. It is pertinent to note that exogenously applied hormones did stimulate the development of proteases and proteolytic processes in detached cotyledons and this stimulation was even higher than that was observed in attached cotyledons.

Discussion

Solvation of insoluble proteins, activation of pre-existing enzymes and/or *de novo* synthesis of enzymes, and degradation of storage proteins are apparently a chain of events leading to the transport of products to the growing axis for the

Table 1. Effect of 1% casein hydrolysate on protein mobilization in attached and detached cotyledons during germination. The attached and detached cotyledons were incubated in water or 1% casein hydrolysate solution for the time intervals indicated and analyzed for protein, FAA (Free amino acids) and protease activities. Each value is mean ±SE of five values.

Germination Time (Days)	Protein (mg/2 cotyledons)		FAA (mg/2 cotyledons)		Protease(s) activity (μ moles of amino acids released /h/2 cotyledons) Acid		Neutral		Alkaline	
	Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated
Attached cotyledons										
0	30.00±0.62	30.00±0.62	0.72±0.05	0.72±0.05	1022±63	1022±63	950±43	950±43	455±20	456±22
2	19.72±0.64	28.02±0.59	2.20±0.06	6.73±0.5	1928±20	1052±38	1088±58[a]	970±55[a]	548±43[b]	576±44[b]
4	18.30±0.63	24.78±0.40	2.46±0.10	6.63±0.4	2592±92	1084±96	1344±40	1032±47	1308±88	434±60
6	16.40±0.78	22.69±0.42	2.98±0.04	5.00±0.5	1699±66	704±93	863±32	704±36	640±64	394±38
8	12.80±0.45	19.24±0.36	2.34±0.06	3.53±0.3	1129±59	674±92	584±43	464±52	456±58	242±25
10	8.26±0.12	16.80±0.61	1.24±0.04	2.53±0.2	790±30	372±30	494±14	264±25v	413±13	164±18
Detached cotyledons										
0	30.00±0.62	30.00±0.62	0.72±0.05	0.72±0.05	1022±63	1022±63	950±43	950±43	455±20	456±22
2	24.12±0.54	30.10±0.9	2.38±0.05	6.19±0.34	1803±64	834±48	1314±43	716±40	488±35[c]	466±26[c]
4	22.28±0.70	27.50±0.5	2.96±0.09	6.83±0.74	2086±132	648±54	1474±42	796±92	956±70	480±51
6	20.08±0.31	25.80±0.5	3.57±0.05	7.12±0.56	2292±74	496±33	1244±63	536±45	743±13	460±59
8	17.98±0.80	24.92±0.5	4.03±0.04	8.51±0.50	1362±84	280±25	1180±23	440±23	535±23	392±37
10	14.98±0.52	23.15±0.9	4.10±0.03	9.53±0.60	894±64	196±27	1028±25	286±25	505±14	322±22

Mean ± SE followed by the same letter do not differ according to DMR test at 5% level of significance ($P < 0.01$).

synthesis of new proteins and other nitrogenous compounds (Bewley and Black 1994; Callis 1995; Shewry et al. 1995). Consistent with these general phenomena, we (Ramakrishna and Ramakrishna Rao 2005) reported previously that the total protein of Indian bean is depleted during germination period. The decrease in protein content in the cotyledons during germination is also reported in lima bean (Heywood and Gainer 1974), horse gram (Karunagaran and Ramakrishna Rao 1990; Rajeswari and Ramakrishna Rao 2002), lupine (Nandi et al. 1995), *Vigna mungo* (Taneyama et al. 1996) and *Vicia sativa* (Misra and Kar 1990). However, in the cotyledons of vetch during germination the amounts of the amino acids and proteins did not change (Schlereth et al. 2001). The existence and development of acid, neutral and alkaline proteases have also been noted in the cotyledons of germinating legume and non-legume seeds (Misra and Kar 1990; Shastry and John 1991). Increase in proteolytic activity with concomitant reserve protein depletion agrees with the findings of earlier works in other legume seeds: *Phaseolus vulgaris* (Senyuk et al. 1998), *Vigna mungo* (Taneyama et al. 1996), *Lupinus albus* (Ferreira et al. 1995), *Vicia sativa* (Schlereth et al. 2001), and horse gram (Karunagaran and Ramakrishna Rao 1990; Rajeswari and Ramakrishna Rao 2002).

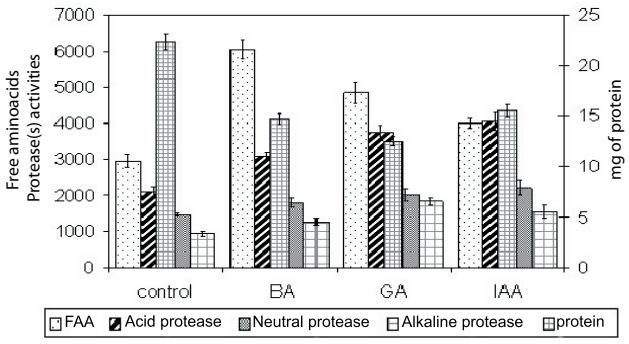

Figure 3. Changes in the levels of protein, free amino acids and activities of proteases (acid, neutral and alkaline) in detached cotyledons incubated in the presence of hormone (0.01 mM) for 4 days compared with corresponding controls incubated in water.

The regulation of proteolysis in the Indian bean cotyledons by feedback inhibition by the end products is favored by several observations made in this study: (1) the amino acid content in detached cotyledons increases continuously during the time course of study and the depletion of protein and the development of proteases are retarded compared with attached cotyledons (Figs. 2A, 2B); (2) the activities of proteases are also retarded when the cotyledons (attached and detached) are incubated with casein hydrolysate substantiating the inverse relationship noticeable between the amino acid content and the activities of proteases in detached cotyledons (Table 1). In agreement with these results, the protein mobilization in buckwheat is subject to feedback inhibition by accumulation of protein degradation products *in vitro* and *in vivo* (Dunavsky and Belozersky 1993). Treatment with casaminoacids inhibited the growth of mung bean seedlings with a parallel inhibition of the development of vacillin peptidohydrolase activity in the cotyledons (Kern and Chrispeels

1978). The findings of the present study support the notion that the feedback regulation is highly likely to be involved in the control of protease development during the germination of Indian bean.

Exogenously applied plant growth hormones (BA, GA_3 or IAA) in the present study stimulated the development of proteases and proteolysis in detached cotyledons indicating the possibility of involvement of hormones in the axial control of development of proteases. In line with present data, Taneyama et al. (1996) showed that the levels of endopeptidase activity was doubled when GA_3 (10-100 μM) was applied to the detached cotyledons of *Vigna mungo* seeds and they suggested the possibility that gibberellins (GA_3) synthesized in axis of seedlings are transported to cotyledons and that the biologically active from GA_3 triggers the expressions of SH-EP in cotyledons. Similarly in lupine (Nandi et al. 1995) and squash cotyledons (Ashton 1976) the plant hormone, cytokinin could substitute for the embryonic axis with respect to endopeptidase development. The effect of the removal of the axis on the protease level might be ascribable to the removal of endogenous source of plant hormones that might stimulate the synthesis of the enzyme in the cotyledons.

In conclusion, the results obtained from this study indicate that the embryonic axis is required for reserve protein mobilization in the cotyledons of germinating Indian bean. The two hypotheses, source-sink and hormonal stimulus were influencing in the mobilization of food reserves and the growth of seedling. While the phytohormone signals transmitted from the axis to the storage tissue appear to be necessary for the development of proteases and regulated their activity *in vivo via* feedback mechanism by the levels of end products at the site of reserve food breakdown. The hormonal effect on protease may be due to the induction of enzyme synthesis or suppression of the degradation of enzyme or both. Further investigation into these possibilities is in progress to identify whether the hormonal effect is through induction at early stages of germination or due to slower degradation of protease in the later stages of germination.

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